

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tsang et al.

Art Unit: not yet assigned

Application No. 10/048,146

Filed: January 25, 2002

For: METHODS AND COMPOSITIONS FOR
DETECTING LARVAL TAENIA SOLIUM

Examiner: not yet assigned

Date: April 10, 2002

BOX PCT
COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service on April 10, 2002 as First Class Mail in an envelope addressed to: BOX PCT, COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.


Agent for Applicants

SECOND PRELIMINARY AMENDMENT

Sequence Listing

Please replace the sequence listing with the enclosed replacement sequence listing (seven pages).

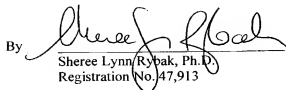
REMARKS

By this Amendment, the sequence listing has been replaced with a corrected sequence listing to include the present application number and to simplify the names of the inventors. A new sequence listing is submitted due to an apparent problem with the computer readable version previously submitted.

No new matter has been added by this Amendment. If there are any questions about this Amendment, the examiner is invited to telephone the undersigned.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By 
Sheree Lynn Rybak, Ph.D.
Registration No. 47,913

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121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

1

SEQUENCE LISTING

<110> Tsang, Victor C. W.
Greene, Ryan M.
Wilkins, Patricia P.
Hancock, Kathy

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<130> 03063-0551WP

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WO 01/10897

PCT/US00/21173

4

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WO 01/10897

PCT/US00/21173

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WO 01/10897

PCT/US00/21173

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10048146 .012502

JC07 R3cd PCT/PTO 25 JAN 2002

FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 6395-62068
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. § 1.5) 10/048146
INTERNATIONAL APPLICATION NO. PCT/US00/211173	INTERNATIONAL FILING DATE August 3, 2000	PRIORITY DATE CLAIMED August 5, 1999
TITLE OF INVENTION METHODS AND COMPOSITIONS FOR DETECTING LARVAL <i>TAENIA SOLIUM</i>		
APPLICANT(S) FOR DO/EO/US Victor C.W. Tsang, Ryan M. Greene, Patricia P. Wilkins, Kathy Hancock		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371. 3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. § 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. § 371(c)(2)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is transmitted herewith (attached to the Declaration). b. <input type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. § 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)). 		
Items 11. to 16. below concern document(s) or information included:		
<ol style="list-style-type: none"> 11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98, with copies of 14 references cited. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§ 3.28 and 3.31 and the Recordal fee of \$40.00 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment, including an Abstract on a separate page. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information: <ul style="list-style-type: none"> <input checked="" type="checkbox"/> Written Opinion. <input checked="" type="checkbox"/> Preliminary Examination Report. <input checked="" type="checkbox"/> International Search Report. <input checked="" type="checkbox"/> Replacement Sequence Listing <ul style="list-style-type: none"> <input checked="" type="checkbox"/> Paper copy (7 pages) <input checked="" type="checkbox"/> Computer Readable Form (diskette) <input checked="" type="checkbox"/> Statement in Compliance 		



531 Rec'd PCT/APP-25 JAN 2002

EXPRESS MAIL LABEL NO. EV053213529US

DATE OF DEPOSIT: January 25, 2002

U.S. APPLICATION NO. 10/048146		INTERNATIONAL APPLICATION NO. PCT/US00/21173		ATTORNEY'S DOCKET NUMBER 6395-62068	
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 C.F.R. §§ 1.492(a)(1)-(5)): Neither International Preliminary Examination fee (37 C.F.R. § 1.482) nor International Search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1,040.00 International Preliminary Examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$890.00 International Preliminary Examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO as an International Searching Authority.....\$740.00 International Preliminary Examination fee paid to USPTO (37 C.F.R. § 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$710.00 International Preliminary Examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00				CALCULATIONS (PTO USE ONLY)	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$	890.00
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 C.F.R. § 1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	16 - 20 =	0	x \$18.00	\$	0.00
Independent Claims	5 - 3 =	2	x \$84.00	\$	168.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$	1,058.00
<input type="checkbox"/> Reduction of 1/2 for filing by small entity. Small entity status is claimed for this application.				\$	
SUBTOTAL =				\$	1,058.00
Processing fee of \$130.00 for furnishing the English translation later than Months from the earliest claimed priority date (37 C.F.R. §§ 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30				\$	
TOTAL NATIONAL FEE =				\$	1,058.00
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). \$40.00 per property.				\$	
TOTAL FEES ENCLOSED =				\$	1,058.00
				REFUND →	\$
				CHARGE →	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ 1,058.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Director is hereby authorized to charge any additional fees that may be required, or credit any overpayment, to Deposit Account No. 02-4550. A duplicate copy of this sheet is enclosed. d. <input checked="" type="checkbox"/> Please return the enclosed postcard to confirm that the items listed above have been received.					
NOTE: Where an appropriate time limit under 37 C.F.R. § 1.494 or § 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: KLARQUIST SPARKMAN, LLP One World Trade Center, Suite 1600 121 S.W. Salmon Street Portland, OR 97204-2988					
SIGNATURE Tanya M. Harding, Ph.D. NAME 42,630 REGISTRATION NUMBER					

cc: Docketing

531 Rec'd PCT 25 JAN 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Tsang *et al.*

Art Unit: Not yet assigned

Application No. Not yet assigned

Filed: Herewith

For: METHODS AND COMPOSITIONS FOR
DETECTING LARVAL TAENIA SOLIUM

Examiner: Not yet assigned

Date: January 25, 2002

BOX PCT
COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

PRELIMINARY AMENDMENT

In the Specification:

Please insert the following header and paragraph on page 1, immediately following the title:

--CROSS REFERENCE TO RELATED APPLICATIONS

This is a §371 U.S. National Stage of International Application No. PCT/US00/21173, filed August 3, 2000, (published in English under PCT Article 12(2)), which in turn claims the benefit of U.S. Provisional Application No. 60/147,318, filed August 5, 1999. --

Please insert the enclosed Abstract of the Disclosure at the end of the application, on a separate page.

Sequence Listing:

Please replace the sequence listing from the international application with the enclosed replacement sequence listing (seven pages).

REMARKS

By this Amendment, the specification has been changed to relate to prior related applications. Applicants submit herewith an Abstract of the Disclosure on a separate page. This Abstract is identical to the abstract on the cover sheet of the corresponding PCT application.

In addition, the sequence listing has been replaced with a corrected sequence listing to correct an obvious typographical error in the original sequence as it was filed. The error (corrected) is shown in the attached Marked up Version of the Sequence Listing.

No new matter has been added by these Amendments.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By

Tanya M. Harding, Ph.D.
Registration No. 42,630

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121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

531 Rec'd PCIT 25 JAN 2002

**Marked-up Version of Amended Sequence Listing
Pursuant to 37 C.F.R. §§ 1.121(b)-(c)**

In the description of SEQ ID NO: 7, on page 6 of the replacement sequence listing:

<222> (20)..(21)

<223> Amino acid at position ~~19~~20 may also be arginine

ABSTRACT OF THE DISCLOSURE**METHODS AND COMPOSITIONS FOR DETECTING LARVAL *TAENIA SOLIUM***

Compositions and methods for the detection of *Taenia solium* and the diagnosis and treatment of *T. solium* infection are described. The nucleotide and amino acid sequences of the antigenic polypeptides TS-14, TS-18 and TSRS-1 are provided. The compositions contain antigenic polypeptides of larval origin. The polypeptides are useful as research tools for studying *T. solium* and as reagents in assays for the detection of *T. solium* antibodies in a biological sample. The methods are sensitive and specific assays that utilize the antigenic polypeptides or nucleic acid molecules encoding the larval polypeptides.

531 Rec'd PCT

25 JAN 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Tsang *et al.*

Art Unit: Not yet assigned

Application No. Not yet assigned

Filed: Herewith

For: METHODS AND COMPOSITIONS FOR
DETECTING LARVAL TAENIA SOLIUM

Examiner: Not yet assigned

Date: January 25, 2002

STATEMENT IN COMPLIANCE WITH 37 C.F.R. § 1.821(f)

BOX PCT
COMMISSIONER FOR PATENTS
WASHINGTON, DC 20231

Sir:

In compliance with 37 C.F.R. § 1.821(f), the undersigned declares that the nucleotide and/or amino acid sequences presented in the paper copy of the "Sequence Listing" submitted herewith are the same as the sequences contained in the computer-readable form of the "Sequence Listing."

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

By

Tanya M. Harding, Ph.D.
Registration No. 42,630

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 226-7391
Facsimile: (503) 228-9446

SEQUENCE LISTING

<110> The Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, Centers for Disease Control and Prevention

Tsang, Victor
Greene, Ryan
Wilkins, Patricia
Hancock, Kathy

<120> METHODS AND COMPOSITIONS FOR DETECTING LARVAL TAENIA SOLIUM

<130> 6395-62068

<150> US 60/147,318

<151> 1999-08-03

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Ser Val His Ile Ala Ser Cys Lys Ser His Tyr Met Phe Gln Leu Lys
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cgc ttt ttt gcc ttt agg aaa aac aaa ccg aaa gat gtt gca aat agt      315
Arg Phe Phe Ala Phe Arg Lys Asn Lys Pro Lys Asp Val Ala Asn Ser
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acg aaa aaa ggg ata gaa tat gtc cac gaa ttc ttc cac gaa gac ccg      363
Thr Lys Lys Gly Ile Glu Tyr Val His Glu Phe Phe His Glu Asp Pro
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att ggt aaa caa att gct caa ctc gca aag gaa tgg aag gaa gca atg      411

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Ile His Leu Asp Lys Gly Lys Ile Arg Thr Ser Leu Val Glu His Cys	
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Lys Gly Pro Lys Lys Lys Thr Ala	
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Lys Ser His Tyr Met Phe Gln Leu Lys Arg Phe Phe Ala Phe Arg Lys
 35 40 45

Asn Lys Pro Lys Asp Val Ala Asn Ser Thr Lys Lys Gly Ile Glu Tyr
 50 55 60

Val His Glu Phe Phe His Glu Asp Pro Ile Gly Lys Gln Ile Ala Gln
 65 70 75 80

Leu Ala Lys Glu Trp Lys Glu Ala Met Leu Glu Gly Arg Phe Trp Cys
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 Ala Asn Ser Thr Lys Lys Glu Ile Glu Tyr Ile His Asn Trp Phe Phe
 20 25 30
 cat gat gac ccg att gga aaa caa att gct caa ctg gca aag gac tgg 143
 His Asp Asp Pro Ile Gly Lys Gln Ile Ala Gln Leu Ala Lys Asp Trp
 35 40 45
 aat gaa aca gtg cag gaa gcc aaa gcc aaa ttt tgg gcg tca ctg gct 191
 Asn Glu Thr Val Gln Glu Ala Lys Gly Lys Phe Trp Ala Ser Leu Ala
 50 55 60
 gag tac tgc aga ggt ctg aag aac aaa act gct taacttgtca actttcatgc 244
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 Val Lys Asn Ile Lys Lys Gly Met Glu Val Val Tyr Lys Phe Phe Tyr
 20 25 30
 gaa gac ccg ttg gga aag aaa ata gct caa ctc gca aag gac tgg aag 143
 Glu Asp Pro Leu Gly Lys Lys Ile Ala Gln Leu Ala Lys Asp Trp Lys
 35 40 45
 gaa gca atg ttg gaa gcc aga agc aaa gtg cgg gcg tca ctg gct gag 191
 Glu Ala Met Leu Glu Ala Arg Ser Lys Val Arg Ala Ser Leu Ala Glu
 50 55 60
 tac atc aga ggt ctc aag aac gaa gct gct taacttgta actttcatgc 241
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1 5

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<400> 9

Gly Ile Glu Tyr Val His Glu Trp Phe Phe His Glu Asp
 1 5 10

531 Rec'd PCT

25 JAN 2002

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METHODS AND COMPOSITIONS FOR
DETECTING LARVAL TAENIA SOLIUM

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This invention was made by the Centers for Disease Control and Prevention, an agency of the United States Government. Therefore, the United States Government has certain rights in this invention.

FIELD OF THE INVENTION

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The present invention relates to the fields of molecular biology and immunology and more specifically relates to compositions and methods for diagnosing cysticercosis. In particular, the invention pertains to synthetic or recombinant *Taenia solium* antigens and their use in immunoassays.

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BACKGROUND OF THE INVENTION

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Taenia solium cysticercosis, caused by infection with *T. solium* larval cysts, occurs in both humans and swine, resulting in significant public health and economic hardship. *T. solium*, also referred to as the pork tapeworm, is a helminth that exists in both a mature tapeworm form and a larval form. The lifecycle of *T. solium* begins when swine, the intermediate hosts, ingest tapeworm eggs excreted in the feces of a tapeworm carrier. The larvae hatch from the eggs and invade most tissues of the swine, giving rise to the disease cysticercosis.

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When humans ingest raw or undercooked meat from cysticercotic swine, tapeworms, or taeniasis, develop. Patients with taeniasis may exhibit epigastric discomfort, nausea, irritability, diarrhea and weight loss. In addition, proglottids, or individual segments of the

2

tapeworm that are self-contained hermaphroditic reproductive units, may obstruct the appendix, biliary duct, or pancreatic duct.

Humans may also ingest *T. solium* eggs present in contaminated food and water and serve as intermediate hosts. After *T. solium* eggs are ingested, cysticerci may develop in the subcutaneous tissues, muscles, heart, lungs, liver, brain, and eye. Although small numbers of viable cysticerci fail to produce symptoms in the infected host, death of the larvae stimulate a marked inflammatory reaction, fever, muscle pains, and eosinophilia. If the larvae invade the central nervous system, the host may develop meningoencephalitis, epileptic seizures, dementia and other neurologic or psychiatric manifestations, and can result in death from acute intracranial hypertension. The various manifestations of neurologic dysfunction caused by *T. solium* infection are collectively termed neurocysticercosis. Although neurocysticercosis can include many neurological symptoms, epilepsy is the most common symptom. In fact, *T. solium* is considered the leading infectious cause of epileptic seizures worldwide. Additionally, *T. solium* neurocysticercosis has a current worldwide toll of 50 million cases with 50,000 deaths each year.

Neurocysticercosis is rarely acquired in the United States; however, the disease is common in Latin America, Asia, Russia and Eastern Europe. In Mexico, the mean rate for cysticercotic pigs in inspected slaughterhouses during 1980-1981 was 1.55%, and in rural areas of Mexico and South America where sewage disposal is limited, the number of cysticercotic pigs can be in excess of 50%. In these and other developing countries, the parasite causes a substantial economic burden to the pork industry. Additionally, due to the increased travel and immigration from highly endemic areas, detection and treatment of *T. solium* related diseases has become a U.S. public health priority.

Diagnosis historically relied on histological identification of the parasite by biopsy or autopsy. The recent development of radiologic and serologic methods has improved diagnosis. However, while radiologic methods such as computed tomography (CT) or nuclear magnetic resonance imaging are useful in diagnosing neurocysticercosis, they are often too expensive or inaccessible in developing countries. Although some diagnostic tests are currently available to identify *T. solium* infection and diagnose neurocysticercosis, these tests, such as the one described in U.S.

Patent No. 4,740,456 to Kuhn *et al.*, lack specificity and sensitivity. A more specific and sensitive assay for diagnosing human neurocysticercosis by detecting the presence of *T. solium* larvae using immunoelectrotransfer blot (EITB) is described in U.S. Patent No. 5,354,660 to Tsang *et al.* However, the assay utilizes purified, naturally-occurring *T. solium* larval glycoproteins, not recombinantly produced antigens, thereby making the assay reagents expensive and difficult to produce. In developing countries where *T. solium* related diseases are endemic, access to diagnostic assays may be limited due to the high cost of using antigens produced using a complicated purification procedure. Furthermore, because cysticercosis is most prevalent in rural areas of developing countries, a field test is needed for epidemiological studies and surveillance. A field assay would be an important tool in breaking the transmission cycle of the parasite, enabling the on-site diagnosis of infected pigs and immediate treatment with anti-helminthic agents such as oxfendazole. A field diagnosis of cysticercosis would also serve as an economic benefit to pig farmers, because uninfected pigs command a higher price. Therefore there is a need for sensitive, specific, and inexpensive immunoassays containing stable reagents that can detect and measure larval *T. solium* in the clinic, laboratory, and field.

SUMMARY OF THE INVENTION

Compositions and methods for detecting and diagnosing *Taenia solium* are provided. The compositions contain recombinant or synthetic *T. solium* larval polypeptides. The polypeptides are useful in immunoassays for the detection of larval *T. solium* in biological samples. The polypeptides are recombinantly or synthetically produced larval antigens having the nucleic acid sequences provided herein and molecular weights of approximately 14 kDa, 18 kDa and 21 kDa, as determined by SDS-PAGE analysis, or antigenic fragments thereof. The corresponding, naturally occurring polypeptides are subunits of larger *T. solium* glycoproteins and are referred to herein as TS-14, TS-18 and TSRS-1, respectively.

The 14 kDa recombinant larval polypeptide is preferably encoded by the nucleic acid sequence of SEQ ID NO:1 and has the amino acid sequence of SEQ ID NO:2. The 18 kDa recombinant larval polypeptide is preferably encoded by the nucleic acid sequence of SEQ ID NO:3 and has

the amino acid sequence of SEQ ID NO:4. The 21 kDa recombinant larval polypeptide is preferably encoded by the nucleic acid sequence of SEQ ID NO:5 and has the amino acid sequence of SEQ ID NO:6. Recombinant or synthetic polypeptides having the foregoing amino acid or nucleic acid sequences, or antigenic fragments thereof, are useful in immunoassays for the detection of *T. solium*.

The amino acid sequences provided herein are useful for the synthesis of the antigens or antigenic fragments using well known chemical synthesis techniques.

The nucleic acid molecules encoding the larval antigens are useful for the recombinant production of the antigens and antigen fragments and are also useful as molecular probes or primers for the detection of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) involved in transcription and translation of *T. solium* peptides. These molecular probes or primers provide means to detect and measure *T. solium* larval polypeptides in tissues and cells.

The recombinant or synthetic *T. solium* polypeptides can be used in diagnostic kits to detect the presence and quantity of *T. solium* antibodies, which is diagnostic or prognostic for the occurrence or recurrence of diseases such as cysticercosis and neurocysticercosis. The recombinant *T. solium* polypeptides may also be administered to a human or animal in a pharmaceutical composition to immunize the human or animal against *T. solium* infection, thereby reducing or preventing *T. solium* related disease.

The preferred methods provided herein are immunoassays directed toward the detection of *T. solium* antibodies in biological samples such as biological fluids and tissues of humans and animals. Alternative preferred methods are nucleic acid hybridization or amplification assays directed toward the detection of *T. solium* antigens in biological samples.

In a preferred embodiment, an immunoassay employs one or more of the recombinant or synthetic larval polypeptides, or antigenic fragments thereof, for the detection of anti-larval antibodies in a biological sample. The preferred immunoassay is an immunoblot containing recombinant larval antigens, or antigenic fragments thereof, immunoreactive with anti-*T. solium* antibodies in a biological sample.

Diagnostic and analytical methods and kits may be developed for detection and measurement of *T. solium* antibodies and antigens in a variety of samples. The methods and kits can be in any configuration well known to those of ordinary skill in the art.

5 Accordingly, it is an object of the present invention to provide means for detecting *T. solium* carriers and thus prevent the spread of *T. solium* from one host to another.

10 It is another object of the present invention to provide a method for the detection of *T. solium*, particularly the diagnosis or monitoring of *T. solium* infection in humans and animals, that is inexpensive, sensitive and accurate, with little or no cross-reactivity.

15 It is another object of the present invention to provide a simple, sensitive method for the diagnosis of cysticercosis or neurocysticercosis.

20 It is yet another object of the present invention to provide a rapid, simple, and inexpensive assay for the detection of *T. solium* larvae that has a long shelf life, a short assay time, and stable reagents that can be utilized in the field, and the results can be interpreted without the use of instrumentation or special temperature conditions, which is optimal for use in poor, underdeveloped countries where *T. solium* is often endemic.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

25 DETAILED DESCRIPTION

30 Compositions and methods for detecting *T. solium* infection and diagnosing diseases related to *T. solium* infection are provided. The compositions are recombinant or synthetic immunogenic, or immunodominant, polypeptides of the *T. solium* helminth larvae, namely the polypeptides referred to herein as TS-14, TS-18 and TSRS-1, or antigenic fragments thereof. The nucleic acid sequences and amino acid sequences of the *T. solium* larvae polypeptides are provided.

35 The recombinant *T. solium* polypeptides are useful as diagnostic reagents in the immunoassays described below. The polypeptides are also useful *in vitro* as research tools for studying *T. solium* in general and *T. solium* related diseases such as cysticercosis. Additionally, the

6

polypeptides may be useful in pharmaceutical compositions such as vaccines.

The methods provided herein are assays for the detection or quantitation of anti-*T. solium* antibodies or *T. solium* nucleic acid molecules in a sample such as a human or animal fluid or tissue. The recombinant or synthetic *T. solium* polypeptides, or antigenic fragments thereof, or nucleic acid molecules encoding the *T. solium* polypeptides, or probes and primers thereof, are used as reagents in the assays.

Definitions

The terms "a", "an" and "the" as used herein are defined to mean "one or more" and include the plural unless the context is inappropriate.

The terms "polypeptide", "peptide", and "protein", as used herein, are interchangeable and are defined to mean a biomolecule composed of two or more amino acids linked by a peptide bond.

The term "antigen" refers to an entity or fragment thereof which can induce an immune response in a mammal. The term includes immunogens and regions responsible for antigenicity or antigenic determinants. "Antigenic determinant" refers to a region of a *T. solium* protein recognized by an antibody.

As used herein, the term "complementary DNA primer" means an oligonucleotide which anneals to a nucleic acid molecule in a particular orientation to allow for the synthesis of a nascent DNA strand in the presence of a polymerase under the conditions described herein. Also as used herein, the "condition" or "conditions" under which a DNA strand is synthesized include the presence of nucleotides, cations and appropriate buffering agents in amounts and at temperatures such that the nucleic acid molecule and the DNA primer will anneal and oligonucleotides will be incorporated into a synthesized DNA strand.

As used herein, the term "primer pair" refers to two primers, one having a forward designation and the other having a reverse designation relative to their respective orientations on a double-stranded DNA molecule which consists of a sense and antisense sequence, such that under the amplification conditions described herein, the forward primer anneals to and primes amplification of the sense sequence and the reverse primer anneals to

and primes amplification of the antisense sequence. Primers can be selected for use in the amplification reaction on the basis of having less than 50% G-C content, having minimal complementarity with other primers in the reaction (to minimize the formation of primer dimers) and having T_m values with the range of reaction temperatures appropriate for the amplification method, preferably PCR. In addition, primers can be selected to anneal with specific regions of the DNA or RNA template such that the resulting DNA amplification product ranges in size from 100 to 5000 base pairs in length and most preferably around 300 base pairs in length or longer.

As used herein, the terms "detecting" or "detection" refers to quantitatively or quantitatively determining the presence of the biomolecule under investigation.

By "isolated" is meant a biological molecule free from at least some of the components with which it naturally occurs.

By "probe" is meant a nucleic acid sequence that can be used for selective hybridization with complementary nucleic acid sequences for their detection. The probe can vary in length from about 5 to 100 nucleotides, preferably from about 10 to 50 nucleotides, most preferably about 18 to 24 nucleotides. The terms "probe" or "probes" as used herein are defined to include "primers."

The term "antibodies" as used herein includes monoclonal antibodies, polyclonal, chimeric, single chain, bispecific, simianized, and humanized antibodies as well as Fab fragments, including the products of an Fab immunoglobulin expression library.

Taenia solium Polypeptides and Polypeptide Fragments

The compositions provided herein are recombinant or synthetic *T. solium* larval polypeptides that are immunoreactive with *T. solium* antibodies. *T. solium* antibodies are preferably derived from the sera, saliva, cerebrospinal fluid or urine of patients infected with *T. solium*. Most preferably, the antibodies are derived from *T. solium* patient sera. The recombinant or synthetic polypeptides correspond to naturally occurring glycoproteins having molecular weights of approximately 14 kDa, 18 kDa and 21 kDa. The polypeptides, referred to herein as TS-14, TS-18 and TSRS-1, contain the amino acid sequences provided in the attached Sequence Listing as SEQ ID NOS: 2, 4 and 6, respectively, and are

8

preferably encoded by the nucleic acid sequences set forth in SEQ ID NOS:1, 3 and 5. It will be understood by those skilled in the art that the preferred polypeptides include polypeptide analogs, which are defined herein as antigenic peptides containing amino acid sequences differing from SEQ ID NOS:2, 4, or 6 by an amino acid substitution at any position or having other molecules attached to amino acid functional groups. The polypeptides are highly charged and polar, and are lysine-rich. The lysine residues contribute to relatively high isoelectric points, 9.25, 8.45 and 8.95 for TS-14, TS-18 and TSRS-1, respectively. TS-14 has one potential N-linked glycosylation site and TS-18 contains three sites. TSRS-1 has one amidation site. The TS-14 and TS-18 polypeptides contain one and two cysteine residues, respectively. The preferred polypeptides also include fragments of the polypeptides having the same antigenicity or the functional equivalent thereof, referred to herein as antigenic fragments. Preferably, the antigenic fragments contain amino acid sequences that are homologous or substantially homologous to two or all three of the antigenic polypeptides. More preferably, the antigenic fragments contain amino acid sequences that are homologous or substantially homologous to TS-14 and TS-18 such as, but not limited to, the following sequences: IAQLAK (SEQ ID NO:7), KNKPKDD/VAASTKKE/GIEYI/VW/HH/R(N)FFF (SEQ ID NO:8), GIEYV/IHE/N(W)FFHE/DD (SEQ ID NO:9). Most preferably, the antigenic fragment contains the amino acid sequence set forth in SEQ ID NO:7.

The *T. solium* polypeptides described herein have a variety of uses. For example the polypeptides or polypeptide fragments are used as reagents in immunoassays for the detection of *T. solium* antibodies as described in more detail below. Furthermore, *T. solium* polypeptides may be employed to develop affinity columns for isolating *T. solium* antibodies. Also, polypeptides that bind to *T. solium* antibodies with high specificity and avidity may be labeled with a label or reporter group and employed for visualization and quantitation in the assays described herein using detection techniques such as autoradiographic and membrane binding techniques. The reporter group or label is commonly a fluorescent or radioactive group or an enzyme. Such applications provide important diagnostic and research tools.

Nucleic Acid Molecules

Nucleic acid molecules encoding the *T. solium* larval polypeptides described above and probes or primers that hybridize to the nucleic acid molecules encoding the *T. solium* larval polypeptides are provided. The preferred nucleic acid molecules are those having sequences encoding the larval *T. solium* polypeptides TS-14, TS-18, and TSRS-1, or fragments thereof, and are provided in the attached Sequence Listing as SEQ ID NOS:1, 3, and 5, respectively. The nucleic acid molecules are useful for production of recombinant polypeptides. Because recombinant methods of polypeptide production produce large quantities of polypeptide that require less purification, recombinant polypeptides are often less expensively produced than polypeptides produced using traditional isolation or purification techniques. The nucleic acid sequences encoding the *T. solium* peptides can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism to produce recombinant *T. solium* peptides in accordance with methods well known to those skilled in the art as described in more detail below.

The nucleic acid molecules are also useful as nucleic acid probes or primers for the detection of *T. solium* infection in a biological specimen with high sensitivity and specificity. The probes or primers can be used to amplify or detect *T. solium* larvae nucleic acid molecules in the sample, quantify the amount of *T. solium* in the sample, diagnose infection or determine contamination with *T. solium*, or monitor the progress of therapies used to treat the infection. The nucleic acid molecules described herein are also useful as laboratory research tools to study the organism and the disease and to develop therapies and treatments for the disease.

The nucleic acid probes or primers provided herein selectively hybridize with nucleic acid molecules encoding the peptides described herein or complementary sequences thereof. Hybridization may be achieved under various temperatures and conditions, according to the temperature of dissociation (Td) of the molecules being hybridized and the stringency required for specific binding. The molecules can be hybridized to one another in any order or preferably at the same time. Reaction conditions for hybridization of an oligonucleotide, or polynucleotide, to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides,

and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words more stringent conditions. Under extremely stringent hybridization conditions, only oligomers that are completely complementary to each other will remain hybridized to each other. In general, the longer the sequence or higher the G and C content, the higher the temperature required or salt concentration permitted. Chapter 11 of the well-known laboratory manual of Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity.

If used as primers, the composition preferably includes at least two nucleic acid molecules which hybridize to different regions of the target molecule so as to amplify a desired region. Depending on the length of the probe or primer, the target region can range between 70% complementary bases and full complementarity and still hybridize under stringent conditions. Preferably, the hybridizing nucleic acid probes or primers described herein have at least 70%, 80%, 85%, 90%, 95%, 97%, 98%, and 99% complementarity with the segment of the sequence to which they hybridize. For the purpose of determining the presence of *T. solium*, the degree of complementarity between the hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes is at least enough to distinguish hybridization with a nucleic acid from other organisms.

Each probe or primer is preferably a DNA molecule having a length of 20 to 40 nucleotides. More preferably, the length of the primer is 25 to 35 nucleotides. The most preferred primer length is 27 to 29 nucleotides.

The amplification of the synthesized DNA can be detected by any method for the detection of DNA known in the art such as by Southern blot hybridization assay, by visualization of DNA amplification products of specific molecular weight on ethidium bromide stained agarose gels, by

11

measurement of the incorporation of radiolabelled nucleotides into the synthesized DNA strand by autoradiography or scintillation measurement, by ELISA modified for the capture of a detectable moiety bound to the amplified DNA, or any other detection method known to one of ordinary skill in the art. The preferred detection method is by hybridization of the amplified DNA to an internal specific oligoprobe using techniques such as ELISA, Southern blot hybridization or similar methods.

The invention contemplates sequences, probes and primers which selectively hybridize to the encoding nucleic acid or the complementary, or opposite, strand of nucleic acid as those specifically provided herein. Specific hybridization with nucleic acid can occur with minor modifications or substitutions in the nucleic acid, so long as functional species-specific hybridization capability is maintained. Isolated nucleic acids are provided herein that selectively hybridize with the nucleic acids encoding the polypeptides under stringent conditions and should have at least five nucleotides complementary to the sequence of interest as described by Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. MOLECULAR CLONING: A LABORATORY MANUAL, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

It will be understood by those skilled in the art that the *T. solium* polypeptides described herein are also encoded by sequences substantially similar to the nucleic acid sequences provided in the Sequence Listing. By "substantially similar" is meant a nucleic acid (including DNA and RNA) sequence which, by virtue of the degeneracy of the genetic code, is not identical with that shown in any of SEQ ID NOS:1, 3 or 5, but which still encodes the same amino acid sequence; or a nucleic acid sequence which encodes a different amino acid sequence but retains the activities or antigenicity of the proteins, either because one amino acid is replaced with another similar amino acid, or because the change (whether it be substitution, deletion or insertion) does not effect the active site of the protein.

Production of Synthetic or *T. solium* Larvae Polypeptides

The nucleic acid sequences provided herein are useful for the production of the proteins or peptides that they encode, or antigenic fragments thereof, by either recombinant or synthetic methods known to

12

those skilled in the art. For example, one or more of the nucleotide sequence provided herein, or a homologue or functional equivalent or portion thereof, can be inserted into a vector, such as a plasmid, and recombinantly expressed in a living organism to produce recombinant polypeptides. Alternatively, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., see Creighton, 1983, PROTEINS-
5 STRUCTURES AND MOLECULAR PRINCIPLES, W. H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequence (e.g., the Edman degradation procedure; see Creighton, 1983, PROTEINS, STRUCTURES AND
10 MOLECULAR PRINCIPLES, W. H. Freeman and Co., N.Y., pp. 34-49).

Recombinant proteins are produced by methods well known to those skilled in the art. A cloning vector, such as a plasmid or phage
15 DNA is cleaved with a restriction enzyme, and the nucleic acid sequence encoding the proteins or fragments thereof of interest is inserted into the cleavage site and ligated. The cloning vector is then inserted into a host to produce the protein or fragment encoded by the nucleic acid. Suitable hosts include bacterial hosts such as *Escherichia coli*, *Bacillus subtilis*, yeasts
20 plants, baculovirus, and other cell cultures. Yeasts are the preferred hosts for vaccine or pharmaceutical product expression. Production and purification of the gene product may be achieved and enhanced using known molecular biology techniques. Mosaic peptides may also be produced by combining various nucleic acid sequences in a cloning vector.

25 Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, GUIDE TO MOLECULAR CLONING TECHNIQUES, METHODS IN ENZYMOLOGY volume 152 Academic Press, Inc., San Diego, CA; Sambrook *et al.* (1989)
30 MOLECULAR CLONING - A LABORATORY MANUAL (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement).
35 Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological

methods. Such manufacturers include the SIGMA Chemical Company (Saint Louis, MO), R&D Systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen (Carlsbad, CA), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

Provided with the peptide sequences described herein, one of skill will recognize a variety of equivalent nucleic acids that encode the peptides. This is because the genetic code requires that each amino acid residue in a peptide is specified by at least one triplet of nucleotides in a nucleic acid which encodes the peptide. Due to the degeneracy of the genetic code, many amino acids are equivalently coded by more than one triplet of nucleotides. For instance, the triplets CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is to be encoded by a nucleic acid triplet, the nucleic acid has any of the triplets which encode arginine. One of skill is thoroughly familiar with the genetic code and its use. An introduction to the subject is found in, for example, chapter 15 of Watson, *et al.*, MOLECULAR BIOLOGY OF THE GENE (Fourth Edition, The Benjamin/Cummings Company, Inc., Menlo Park, CA (1987)), and the references cited therein.

Although any nucleic acid triplet or codon which encodes an amino acid can be used to specify the position of the amino acid in a peptide, certain codons are preferred. It is desirable to select codons for elevated expression of an encoded peptide, for example, when the peptide is purified for use as an immunogenic reagent. Codons are selected by reference to species codon bias tables, which show which codons are most typically used by the organism in which the peptide is to be expressed. The codons used frequently by an organism are translated by the more abundant t-RNAs in the cells of the organism. Because the t-RNAs are abundant, translation of the nucleic acid into a peptide by the cellular translation machinery is facilitated. Codon bias tables are available for most organisms. For an introduction to codon bias tables, *see, e.g.*, Watson, *et al.*, *supra*.

14

In addition, it will be readily apparent to those of ordinary skill in the art that the peptides described herein and the nucleic acid molecules encoding such immunogenic peptides can be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, *i.e.*, to increase biological activity.

One of skill will appreciate that many conservative variations of nucleic acid constructs yield a functionally identical construct. For example, due to the degeneracy of the genetic code, silent substitutions (*i.e.*, substitutions of a nucleic acid sequence which do not result in an alteration in an encoded peptide) are an implied feature of every nucleic acid sequence which encodes an amino acid. In addition, one of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See*, Gilman and Smith (1979) *Gene* 8:81-97, Roberts *et al.* (1987) *Nature* 328:731-734 and Sambrook, Ausbel, Berger and Kimmel, *all supra*.

Modifications to nucleic acids are evaluated by routine screening techniques in suitable assays for the desired characteristic. For instance, changes in the immunological character of encoded peptides can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a complementary nucleic acid, redox or thermal stability of encoded proteins, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

Similarly, conservative amino acid substitutions, in one or a few amino acids in an amino acid sequence of a protein are substituted with different amino acids with highly similar properties (*see*, the definitions section, *supra*), are also readily identified as being highly similar to a disclosed construct. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, *e.g.*, one hydrophobic residue for another, or one polar residue for

another. The substitutions include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are a feature of the present invention.

5 Various techniques for preparing synthetic polypeptides can be used. Solid phase synthesis in which the C-terminal amino acid of the peptide sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for preparing the synthetic peptides. Techniques for solid phase
10 synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*, in *The Peptides: Analysis, Synthesis, Biology* (Gross and Meienhofer (eds.), Academic Press, N.Y., vol. 2, pp. 3-284 (1980)); Merrifield, *et al.*, *J. Am. Chem. Soc.* 85, 2149-2156 (1963); and Stewart,
15 *et al.*, *Solid Phase Peptide Synthesis* (2nd ed., Pierce Chem. Co., Rockford, Ill. (1984)), the teachings of which are hereby incorporated by reference. Many automated systems for performing solid phase peptide synthesis are commercially available.

Solid phase synthesis is started from the carboxy-terminal end (*i.e.*, the C-terminus) of the peptide by coupling a protected amino acid
20 via its carboxyl group to a suitable solid support. The solid support used is not a critical feature of the present invention provided that it is capable of binding to the carboxyl group while remaining substantially inert to the reagents utilized in the peptide synthesis procedure. For example, a starting material can be prepared by attaching an amino-protected amino acid via a
25 benzyl ester linkage to a chloromethylated resin or a hydroxymethyl resin or via an amide bond to a benzhydrylamine (BHA) resin or p-methylbenzhydrylamine (MBHA) resin. Materials suitable for use as solid supports are well known to those of skill in the art and include, but are not limited to, the following: halomethyl resins, such as chloromethyl resin or
30 bromomethyl resin; hydroxymethyl resins; phenol resins, such as 4-(a-[2,4-dimethoxyphenyl]-Fmoc-aminomethyl)phenoxy resin; tert-alkyloxycarbonyl-hydrazidated resins, and the like. Such resins are commercially available and their methods of preparation are known to those of ordinary skill in the art.

35 The acid form of the peptides may be prepared by the solid phase peptide synthesis procedure using a benzyl ester resin as a solid

support. The corresponding amides may be produced by using benzhydrylamine or methylbenz-hydrylamine resin as the solid support. Those skilled in the art will recognize that when the BHA or MBHA resin is used, treatment with anhydrous hydrofluoric acid to cleave the peptide from the solid support produces a peptide having a terminal amide group.

The α -amino group of each amino acid used in the synthesis should be protected during the coupling reaction to prevent side reactions involving the reactive α -amino function. Certain amino acids also contain reactive side-chain functional groups (e.g., sulfhydryl, amino, carboxyl, hydroxyl, etc.) which must also be protected with appropriate protecting groups to prevent chemical reactions from occurring at those sites during the peptide synthesis. Protecting groups are well known to those of skill in the art.

A properly selected α -amino protecting group will render the α -amino function inert during the coupling reaction, will be readily removable after coupling under conditions that will not remove side chain protecting groups, will not alter the structure of the peptide fragment, and will prevent racemization upon activation immediately prior to coupling. Similarly, side-chain protecting groups must be chosen to render the side chain functional group inert during the synthesis, must be stable under the conditions used to remove the α -amino protecting group, and must be removable after completion of the peptide synthesis under conditions that will not alter the structure of the peptide.

Coupling of the amino acids may be accomplished by a variety of techniques known to those of skill in the art. Typical approaches involve either the conversion of the amino acid to a derivative that will render the carboxyl group more susceptible to reaction with the free N-terminal amino group of the peptide fragment, or use of a suitable coupling agent such as, for example, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIPCDI). Frequently, hydroxybenzotriazole (HOBt) is employed as a catalyst in these coupling reactions. Appropriate synthesis chemistries are disclosed in THE PEPTIDES: ANALYSIS, STRUCTURE, BIOLOGY, VOL. 1: METHODS OF PEPTIDE BOND FORMATION (Gross and Meienhofer (eds.), Academic Press, N.Y. (1979)); and Izumiya, *et al.*, SYNTHESIS OF PEPTIDES (Maruzen Publishing Co., Ltd., (1975)).

17

Generally, synthesis of the peptide is commenced by first coupling the C-terminal amino acid, which is protected at the N-amino position by a protecting group such as fluorenylmethyloxycarbonyl (Fmoc), to a solid support. Prior to coupling of Fmoc-Asn, the Fmoc residue has to be removed from the polymer. Fmoc-Asn can, for example, be coupled to the 4-(a-[2,4-dimethoxyphenyl]-Fmoc-amino-methyl)phenoxy resin using N,N'-dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt) at about 25°C for about two hours with stirring. Following the coupling of the Fmoc-protected amino acid to the resin support, the α -amino protecting group is removed using 20% piperidine in DMF at room temperature.

After removal of the α -amino protecting group, the remaining Fmoc-protected amino acids are coupled stepwise in the desired order. Appropriately protected amino acids are commercially available from a number of suppliers (e.g., Novartis (Switzerland) or Bachem (California)). As an alternative to the stepwise addition of individual amino acids, appropriately protected peptide fragments consisting of more than one amino acid may also be coupled to the "growing" peptide. Selection of an appropriate coupling reagent, as explained above, is well known to those of skill in the art. It should be noted that because the immunogenic peptides are relative short in length, this latter approach (i.e., the segment condensation method) is not the most efficient method of peptide synthesis.

Each protected amino acid or amino acid sequence is introduced into the solid phase reactor in excess and the coupling is carried out in a medium of dimethylformamide (DMF), methylene chloride (CH_2Cl_2), or mixtures thereof. If coupling is incomplete, the coupling reaction may be repeated before deprotection of the N-amino group and addition of the next amino acid. Coupling efficiency may be monitored by a number of means well known to those of skill in the art. A preferred method of monitoring coupling efficiency is by the ninhydrin reaction. Peptide synthesis reactions may be performed automatically using a number of commercially available peptide synthesizers (e.g., Biosearch 9500, Biosearch, San Raphael, CA).

The peptide can be cleaved and the protecting groups removed by stirring the insoluble carrier or solid support in anhydrous, liquid hydrogen fluoride (HF) in the presence of anisole and dimethylsulfoxide at about 0°C for about 20 to 90 minutes, preferably 60 minutes; by bubbling

18

hydrogen bromide (HBr) continuously through a 1 mg/10 mL suspension of the resin in trifluoroacetic acid (TFA) for 60 to 360 minutes at about room temperature, depending on the protecting groups selected; or by incubating the solid support inside the reaction column used for the solid phase synthesis with 90% trifluoroacetic acid, 5% water and 5% triethylsilane for about 30 to 60 minutes. Other deprotection methods well known to those of skill in the art may also be used.

The peptides can be isolated and purified from the reaction mixture by means of peptide purification well known to those of skill in the art. For example, the peptides may be purified using known chromatographic procedures such as reverse phase HPLC, gel permeation, ion exchange, size exclusion, affinity, partition, or countercurrent distribution.

Labeled Polypeptides

When labeled with a detectable biomolecule or chemical, the *T. solium* polypeptides and antigenic fragments thereof described above are useful for purposes such as diagnostics and laboratory research using the methods and assays described below. Various types of labels and methods of conjugating the labels to the polypeptides are well known to those skilled in the art. Several specific labels are set forth below.

For example, the polypeptides are conjugated to a radiolabel such as, but not restricted to, ^{32}P , ^3H , ^{14}C , ^{35}S , ^{125}I , or ^{131}I . Detection of a label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the polypeptide by conventional methods, and the labeled polypeptide is detected when an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light.

Fluorogens may also be used as labels. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycoyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The polypeptides can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the

polypeptide can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. Alternatively, the polypeptide can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as LuminoTM) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol) (Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogenic or fluorogenic dioxetane derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, peptides may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art.

The diagnosis of an infection by *T. solium* larvae can be determined by labeling a polypeptide as described above and detecting the label in accordance with methods well known to those skilled in the art.

Detection of *T. solium* Antibodies

Many techniques are known in the art for detecting and quantifying a component such as an antibody in a mixture and/or measuring its amount. Immunoassays, which employ polypeptides that bind specifically to the antibodies of interest, are some of the better known measurement techniques. These methods permit detection of circulating *T. solium* antibodies in order to indicate the presence or level of *T. solium* infection. Classical methods involve reacting a sample containing the antibody with a known excess amount of polypeptide specific for the antibody, separating bound from free antibody, and determining the amount of one or the other. Often the polypeptide is labeled with a reporter group to aid in the determination of the amount of bound analyte as described above. The reporter group or "label" is commonly a fluorescent or radioactive group or an enzyme.

In a preferred embodiment of the present invention, the diagnostic method comprises using an immunoblot assay. In a further preferred embodiment, the diagnostic method is an immunoblot assay

20

containing one or more of the larval *T. solium* glycoprotein antigens referred to herein as TS-14, TS-18 and TSRS-1, or antigenic fragments thereof. As mentioned above, these polypeptides have the amino acid sequences set forth in the Sequence Listing as SEQ ID NOS: 2, 4 and 6, respectively, and are encoded by the nucleic acid sequences set forth in the Sequence Listing as SEQ ID NOS: 1, 3, 5.

It is to be understood that the assay methods are contemplated to include the use of synthetic and recombinant *T. solium* polypeptides as described above and fragments or derivatives of the *T. solium* polypeptides described herein as long as the polypeptide fragments or derivatives retain antigenic activity or display an equivalent antigenic activity of the entire immunogenic polypeptides. These fragments or derivatives include peptides with antigenic activity that have amino acid substitutions or have other molecules attached to amino acid functional groups.

An immunoassay is performed for the detection of *T. solium* in a sample as follows: A sample is collected or obtained using methods well known to those skilled in the art. The sample containing the *T. solium* antibodies to be detected may be obtained from any biological source. Examples of biological sources include blood serum, blood plasma, urine, spinal fluid, saliva, fermentation fluid, lymph fluid, tissue culture fluid and ascites fluid of a human or animal. The sample may be diluted, purified, concentrated, filtered, dissolved, suspended or otherwise manipulated prior to immunoassay to optimize the immunoassay results.

To detect *T. solium* antibodies, the sample is incubated with one or more *T. solium* recombinant or synthetic polypeptides, produced as described above. The polypeptide may be labeled or conjugated to a solid phase bead or particle as also described herein. The labeled polypeptide is then detected using well known techniques for detection of biologic molecules such as immunochemical or histological methods. Such methods include immunological techniques employing monoclonal or polyclonal antibodies to the polypeptide, such as enzyme linked immunosorbant assays, radioimmunoassay, chemiluminescent assays, or other types of assays involving antibodies known to those skilled in the art.

In general, binding assays rely on the binding of analyte by analyte receptors to determine the concentrations of analyte in a sample.

21

These immunoassays can be described as either competitive or non-competitive. Non-competitive assays generally utilize analyte receptors in substantial excess over the concentration of analyte to be determined in the assay. Sandwich assays are examples of non-competitive assays, that comprise one analyte receptor frequently bound to a solid phase and a second analyte receptor labeled to permit detection. The analyte first binds to the analyte receptor bound to a solid phase and the second labeled analyte receptor is then added to facilitate quantitation of the analyte. Bound analyte can easily be separated from unbound reagents, such as unbound labeled first analyte receptors, due to the use of an analyte receptor bound to a solid phase. Competitive assays generally involve a sample suspected of containing analyte, an analyte-analogue conjugate, and the competition of these species for a limited number of binding sites provided by the analyte receptor. Competitive assays can be further described as being either homogeneous or heterogeneous. In homogeneous assays all of the reactants participating in the competition are mixed together and the quantity of analyte is determined by its effect on the extent of binding between analyte receptor and analyte-conjugate or analyte analogue-conjugate. The signal observed is modulated by the extent of this binding and can be related to the amount of analyte in the sample.

In a preferred embodiment, the method for detecting larval *T. solium* antibodies comprises taking biological samples, such as fluids and tissues, from a human or animal for the diagnosis or prognosis of cysticercosis. The sample is preferably obtained from the blood, cerebrospinal fluid, urine, saliva, or tissues of a mammal, preferably a human or pig. A determination of the presence of the antibodies can then be made using assay techniques that are well known to those skilled in the art and include methods such as Western blot analysis, radioimmunoassay and ELISA assays.

Kit for Detecting the Presence of *T. solium*

A kit for detecting the presence and quantity of *T. solium* peptides is also provided. The kit can be in any configuration well known to those of ordinary skill in the art and is useful performing one or more of the methods described herein for the detection of *T. solium* in biological samples or for the detection or monitoring of *T. solium* infection in a patient

or carrier. The kits are convenient in that they supply many if not all of the essential reagents for conducting an assay for the detection of *T. solium* in a biological sample. The reagents may be premeasured and contained in a stable form in vessels or on a solid phase in or on which the assay may be performed, thereby minimizing the number of manipulations carried out by the individual conducting the assay. In addition, the assay may be performed simultaneously with a standard that is included with the kit, such as a predetermined amount of antigen or antibody, so that the results of the test can be validated or measured.

The kit preferably contains one or more *T. solium* polypeptides or nucleic acid molecules that can be used for the detection of *T. solium* antibodies or nucleic acid molecules in a sample. The kit can additionally contain the appropriate reagents for binding the polypeptides to the antibodies or hybridizing the nucleic acid molecules to their respective *T. solium* complementary nucleic acid molecules in the sample as described herein and reagents that aid in detecting the antibody-polypeptide or nucleic acid molecule complexes. The kit may additionally contain equipment for safely obtaining the sample, a vessel for containing the reagents, a timing means, a buffer for diluting the sample, and a colorimeter, reflectometer, or standard against which a color change may be measured.

In a preferred embodiment, the reagents, including the polypeptides, are lyophilized, most preferably in a single vessel. Addition of aqueous sample to the vessel results in solubilization of the lyophilized reagents, causing them to react. Most preferably, the reagents are sequentially lyophilized in a single container, in accordance with methods well known to those skilled in the art that minimize reaction by the reagents prior to addition of the sample.

The assay kit includes but is not limited to reagents to be employed in the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including immunoblots and ELISAs, and immunocytochemistry. Materials used in conjunction with these techniques include, but are not limited to, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood. For each kit, the range,

sensitivity, precision, reliability, specificity and reproducibility of the assay are established.

In a further preferred embodiment, the assay kit uses immunoblot techniques and provides instructions and recombinant larval *T. solium* polypeptides conjugated to a detectable molecule. The kit is useful for the detection and measurement of *T. solium* in biological fluids and tissue extracts of animals and humans to diagnose or monitor cysticercosis or neurocysticercosis.

Immunological and Pharmaceutical Compositions

Immunological compositions, including vaccine, and other pharmaceutical compositions containing the *T. solium* polypeptides or antigenic fragments thereof described herein are useful for reducing or possibly preventing *T. solium* infection or transmission. One or more of the polypeptides described herein are formulated and packaged, alone or in combination with adjuvants or other antigens, using methods and materials known to those skilled in the vaccine art. The immunological response may be used therapeutically or prophylactically and may provide antibody immunity or cellular immunity such as that produced by T lymphocytes such as cytotoxic T lymphocytes or CD4⁺ T lymphocytes.

To enhance immunogenicity, one or more of the polypeptides may be conjugated to a carrier molecule. Suitable immunogenic carriers include proteins, polypeptides or peptides such as albumin, hemocyanin, thyroglobulin and derivatives thereof, particularly bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), polysaccharides, carbohydrates, polymers, and solid phases. Other protein derived or non-protein derived substances are known to those skilled in the art. An immunogenic carrier typically has a molecular weight of at least 1,000 daltons, preferably greater than 10,000 daltons. Carrier molecules often contain a reactive group to facilitate covalent conjugation to the hapten. The carboxylic acid group or amine group of amino acids or the sugar groups of glycoproteins are often used in this manner. Carriers lacking such groups can often be reacted with an appropriate chemical to produce them. Alternatively, a multiple antigenic polypeptide comprising multiple copies of the protein or polypeptide, or an antigenically or immunologically

equivalent polypeptide may be sufficiently antigenic to improve immunogenicity without the use of a carrier.

The *T. solium* polypeptides may be administered with an adjuvant in an amount effective to enhance the immunogenic response against the conjugate. At this time, the only adjuvant widely used in humans has been alum (aluminum phosphate or aluminum hydroxide). Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants used in research and veterinary applications have toxicities which limit their potential use in human vaccines. However, chemically defined preparations such as muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates such as those described by Goodman-Snitkoff *et al.* *J. Immunol.* 147:410-415 (1991), encapsulation of the conjugate within a proteoliposome as described by Miller *et al.*, *J. Exp. Med.* 176:1739-1744 (1992), and encapsulation of the protein in lipid vesicles may also be useful.

The term "vaccine" as used herein includes DNA vaccines in which the nucleic acid molecule encoding *T. solium* polypeptides in a pharmaceutical composition is administered to a patient. For genetic immunization, suitable delivery methods known to those skilled in the art include direct injection of plasmid DNA into muscles (Wolff *et al.*, *Hum. Mol. Genet.* 1:363 (1992)), delivery of DNA complexed with specific protein carriers (Wu *et al.*, *J. Biol. Chem.* 264:16985 (1989)), coprecipitation of DNA with calcium phosphate (Benvenisty and Reshef, *Proc. Natl. Acad. Sci.* 83:9551 (1986)), encapsulation of DNA in liposomes (Kaneda *et al.*, *Science* 243:375 (1989)), particle bombardment (Tang *et al.*, *Nature* 356:152 (1992) and (Eisenbraun *et al.*, *DNA Cell Biol.* 12:791 (1993)), and *in vivo* infection using cloned retroviral vectors (Seeger *et al.*, *Proc. Natl. Acad. Sci.* 81:5849 (1984)).

In a preferred embodiment, a vaccine is packaged in a single dosage for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or nasopharyngeal (i.e., intranasal) administration. The vaccine is most preferably injected intramuscularly into the deltoid muscle. The vaccine is preferably combined with a pharmaceutically acceptable carrier to facilitate administration. The carrier is usually water or a buffered saline, with or without a preservative. The vaccine may be lyophilized for resuspension at the time of administration or in solution.

The carrier to which the polypeptide may be conjugated may also be a polymeric delayed release system. Synthetic polymers are particularly useful in the formulation of a vaccine to effect the controlled release of antigens.

Microencapsulation of the polypeptide will also give a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters polyamides, poly (d,l-lactide-co-glycolide) (PLGA) and other biodegradable polymers.

The preferred dose for human administration of the pharmaceutical composition or vaccine is from 0.01 mg/kg to 10 mg/kg, preferably approximately 1 mg/kg. Based on this range, equivalent dosages for heavier body weights can be determined. The dose should be adjusted to suit the individual to whom the composition is administered and will vary with age, weight and metabolism of the individual. The vaccine may additionally contain stabilizers such as thimerosal (ethyl(2-mercaptobenzoate-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO) or physiologically acceptable preservatives.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

26

Example 1*Expression and Analysis of the 14 and 18 kDa T. solium
Polypeptides*

5 The coding regions for the mature TS-14 and TS-18
polypeptides, set forth in SEQ ID NOS: 1 and 3, were subcloned into the
expression vector pET-32 and expressed in *E. coli* as thioredoxin fusion
proteins. After induction of expression, the total bacterial lysate was
10 analyzed by immunoblot. Total *E. coli* TS-14 and TS-18 proteins from
induced cultures were resolved on SDS-PAGE, blotted onto nitrocellulose,
and probed with cysticercosis infection sera, an anti-thioredoxin monoclonal
antibody (Invitrogen, Carlsbad, CA), antibodies from a serum sample from
an Alaskan native who had an *Echinococcus multilocularis*, and antibodies
15 from a serum pool containing sera from healthy humans residing in the
U.S. with no history of travel. Induced recombinant protein from the
plasmid lacking an insert and encoding only the thioredoxin tag was only
recognized by the anti-thioredoxin monoclonal antibody and migrated at 20
kDa. Anti-cysticercosis antibodies specifically recognized the TS-14 and
20 TS-18 recombinant proteins, which both migrated in SDS-PAGE with a
predicted molecular weight of 28 kDa due to the thioredoxin tag. Neither
subunit reacted with antibodies from the echinococcosis infection serum or
the uninfected serum pool, thereby indicating a lack of cross-reactivity. In
comparing the relative protein concentrations, determined by aurodye
25 staining and by reactivity with the anti-thioredoxin monoclonal antibody,
and anti-cysticercosis reactivity of the 14- and 18-kDa recombinant proteins,
it appears that the 14-kDa recombinant protein is more reactive with anti-
cysticercosis antibodies, consistent with earlier observations of the native
antigen.

30 All of the patents, publications and other references
mentioned herein are hereby incorporated by reference.

 Modifications and variations of the present method will be
obvious to those skilled in the art from the foregoing detailed description.
35 Such modifications and variations are intended to come within the scope of
the appended claims.

CLAIMS

We claim:

- 5 1. A composition comprising one or more isolated, synthetic or recombinant larval *T. solium* polypeptides or antigenic fragments thereof, immunoreactive with *T. solium* antibodies.
- 10 2. The composition of Claim 1 wherein the polypeptides are selected from the group consisting of TS-14, TS-18 and TSRS-1.
- 15 3. The composition of Claim 1 wherein the polypeptides have amino acid sequences selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.
- 20 4. The composition of Claim 1 wherein the polypeptide fragment contains the amino acid sequence set forth in SEQ ID NO:7.
- 25 5. The composition of Claim 1 wherein the polypeptides are encoded by nucleic acid molecules having nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.
- 30 6. An isolated nucleic acid molecule having a sequence encoding a larval *Taenia solium* polypeptide, wherein the nucleic acid sequence encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.
- 35 7. An isolated nucleic acid molecule having a sequence encoding a larval *Taenia solium* polypeptide, wherein the nucleic acid sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.
8. A method for detecting *T. solium* antibodies in a biological sample comprising combining the sample with one or more recombinant or

synthetic larval *Taenia solium* polypeptides, or antigenic fragments thereof, immunoreactive with *T. solium* antibodies and detecting the formation of a complex between the polypeptides or fragments thereof and antibodies in the sample, wherein the presence of an antibody-polypeptide complex indicates the presence of *T. solium* antibodies in the sample.

9. The method of Claim 8 wherein the polypeptides are selected from the group consisting of TS-14, TS-18 and TSRS-1.

10. The method of Claim 8 wherein the polypeptides have amino acid sequences selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

11. The method of Claim 8 wherein the polypeptide fragment contains the amino acid sequence set forth in SEQ ID NO:7.

12. The method of Claim 8 wherein the polypeptides are encoded by nucleic acid molecules having nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

13. A method for diagnosing cysticercosis in a mammal comprising contacting a biological sample of the mammal with one or more synthetic or recombinant larval *Taenia solium* polypeptides, or antigenic fragments thereof, immunoreactive with *T. solium* antibodies, and detecting the binding of antibody present in the biological sample to a *Taenia solium* glycoprotein antigen, wherein the detection of binding indicates cysticercosis.

14. The method of Claim 13 wherein the polypeptides have an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

15. The method of Claim 13 wherein the polypeptide fragment contains the amino acid sequence set forth in SEQ ID NO:7.

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16. The method of Claim 13, wherein the polypeptides are encoded by nucleic acid molecules having nucleic acid sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS AND COMPOSITIONS FOR DETECTING LARVAL TAENIA SOLIUM, the specification of which

- ☒ is attached hereto.
- ☐ was filed on _____ as United States Application No. _____.
- ☒ was filed on August 3, 2000, as International Application No. PCT/US00/21173.
- ☐ and was amended on _____ (if applicable).
- ☐ with amendments through _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56. If this is a continuation-in-part application filed under the conditions specified in 35 U.S.C. § 120 which discloses and claims subject matter in addition to that disclosed in the prior copending application, I further acknowledge the duty to disclose material information as defined in 37 CFR § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of an PCT International application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT International application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application(s)	Priority Claimed
	<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

60/147,318	August 5, 1999
(Application No.)	(Filing Date)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application, to file a corresponding international application, and to transact all business in the Patent and Trademark Office connected therewith:

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application, to file a corresponding international application, and to transact all business in the Patent and Trademark Office connected therewith:

Customer Number:



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all of the law firm of Klarquist Sparkman, LLP; and grant an Associate Power of Attorney to the following:

Name	Reg. No.	Name	Reg. No.
Andrew Watkins	<u>38,653</u>	Russ Metler	<u>45,365</u>
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00
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Inventor's Signature

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As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

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